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Multiplex PCR Assay for Identification of Six Different *Staphylococcus* spp. and Simultaneous Detection of Methicillin and Mupirocin Resistance

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We describe a new, efficient, sensitive, and fast single-tube multiple-PCR protocol for the identification of the most clinically significant *Staphylococcus* spp. and the simultaneous detection of the methicillin and mupirocin resistance loci. The protocol identifies at the species level isolates belonging to *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. lugdunensis*, and *S. saprophyticus*.

Humans are the main natural reservoir of the Gram-positive coagulase-positive bacterium *Staphylococcus aureus* (1). The continuous accumulation of resistance and virulence factors in this species has resulted in a worldwide health concern due to an associated increase in morbidity and mortality (1, 2, 3). Hospital infections by this agent are particularly concerning, especially in those patients who are at risk for complications (i.e., immunocompromised patients, pregnant women, newborns and babies, cancer patients, individuals at dialysis programs, and transplant recipients, etc.) (4). Several methicillin-resistant *S. aureus* (MRSA) clones constitute a global alarm, often being epidemic or even a cause of pandemics (5, 6). However, within the genus *Staphylococcus*, *S. aureus* is not the only species that constitutes a worrisome pathogen. Thus, several coagulase-negative members of the genus are the etiological agents of diverse hospital-acquired severe infections. The most clinically significant examples are the species *S. epidermidis*, *S. saprophyticus*, *S. lugdunensis*, *S. haemolyticus*, and *S. hominis* (7–13). Their pathogenic features can become so hazardous that new, effective antibiotics and efficient, sensitive, and fast diagnostic methods constitute cornerstones in the fight against these adverse bacteria (12–15).

Introduction of novel drugs has always been followed by the prompt appearance of new staphylococcal resistances. Methicillin was introduced in 1959 to overcome the problems that arose from the increasing prevalence of penicillin-resistant *S. aureus* isolates (16). Two years later, MRSA strains were detected. Few antibiotics are still active against MRSA, with mupirocin being one of them. Mupirocin is normally used as a topical agent to prevent MRSA invasion (17, 18). Moreover, it is also a recommended antibiotic for use when invasive surgeries are performed (17). Unfortunately, 2 years after its introduction, high-level mupirocin resistance appeared and has worryingly increased since that time. Such resistance is commonly mediated by a conjugative plasmid-associated locus (*ileS2*) (19). Genetic transfer of *ileS2* plasmids has given rise to mupirocin-resistant *Staphylococcus* clones belonging to several *Staphylococcus* species (20). The growing incidence of staphylococcus-resistant strains has created a need for the availability of *Staphylococcus* identification methods able to detect antibiotic resistance of multiple strains simultaneously, such as the new multiple-PCR (mPCR) protocol described in this study.

Bacterial isolates, identification, and susceptibility testing. A total of 67 clinical isolates were included in this study for the validation of the assay. Initially, 16 isolates were used to test all PCR primer pairs. All of these isolates were recovered from clinical samples from 67 patients at the Microbiology Service of the Hospital Universitario Nuestra Señora de Candelaria (HUNSC). Three *S. aureus* reference strains (ATCC 29213, ATCC 25923, and NCTC8325) were included in the study as well. Before the molecular analysis, all isolates were biochemically identified at the HUNSC Microbiology Service as follows. Clinical isolates were recovered by culturing clinical samples on Columbia agar plates with 5% sheep blood and onto mannitol-salt agar (MSA) plates (bioMérieux, Marcy l'Etoile, France). Plates were incubated at 35 to 37°C for 24 to 48 h under aerobic conditions. Phenotypic identification of the isolates was done based on colony morphology, growth features on MSA, Gram staining, and catalase, coagulase, and DNase tests. Susceptibility testing was performed at the HUNSC Microbiology Service according to CLSI criteria (21, 22). *Staphylococcus* isolates were analyzed with the Vitek 2 system (GPS-511 card) (bioMérieux, Marcy l'Etoile, France). In addition, the susceptibility of the isolates to oxacillin and mupirocin was retested at the HUNSC Research Unit before molecular analysis was performed. Methicillin resistance was confirmed by disk diffusion testing with 1 μg oxacillin, using Mueller-Hinton agar (Difco Laboratories, MI). Intermediate methicillin resistance was confirmed with oxacillin Etest strips (AB Biodisk). Mupirocin re-

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TABLE 1 Primers used in this study

Target identification (locus)	Primer	Sequence (5′–3′)	T_m[a]	Amplicon size (bp)	Reference
S. lugdunensis (fbl)	fblF	AAA TCT CCA AGT TGA CCA AAC ATA C	52.6	550	Pereira et al., 2010 (11)
	fblR	GAT TGC GCT GAA AGA ATT GC	52.6		
Mupirocin resistance (*ileS2*)	ileS2F (mup1)	TAT ATT ATG CGA TGG AAG GTT GG	52.5	456	Pérez-Roth et al., 2001 (20)
	ileS2R (mup2)	AAT AAA ATC AGC TGG AAA GTG TTG	52.5		
S. saprophyticus (sap)	sapF	AAC GGG CGT CTC GAT AGA AAA	56.4	380	Martineau et al., 2000 (8)
	sapR	AAC GGG CGT CCA CAA AAT CA	57.7		
S. aureus (nuc)	nucF	TCG CTT GCT ATG ATT GTG G	52.9	359	Hirotaki et al., 2011 (19)
	nucR	GCC AAT GTT CTA CCA TAG C	50.8		
Methicillin resistance (*mecA*)	mecA1	GTA GAA ATG ACT GAA CGT CCG ATA A	54.6	310	Pérez-Roth et al., 2013 (18)
	mecA2	CCA ATT CCA CAT TGT TTC GGT CTA A	55.7		
S. haemolyticus (mvaA)	mvaA1 (hae1)	GGT CGC TTA GTC GGA ACA AT	54.9	271	Schuenck et al., 2008 (12)
	mvaA2 (hae2)	CAC GAG CAA TCT CAT CAC CT	54.7		
S. epidermidis (sep)	sepF	CAG TTA TAC GGT ATG AGA GC	50.2	219	Hirotaki et al., 2011 (19)
	sepR	CTG TAG AGT GAC AGT TTG GT	51.7		
S. hominis (hom)	homF	TAC AGG GCC ATT TAA AGA CG	52.5	177	Hirotaki et al., 2011 (19)
	homR	GTT TCT GGT GTA TCA ACA CC	51.1		

[a] Optimal annealing temperature.

sistance was screened by the disk diffusion method (Oxoid, Basingstoke, England): 5-μg mupirocin disks were used to detect low-level resistance, and 200-μg disks were used to detect high-level resistance. Finally, confirmation of high-level resistance was performed with Etest strips (AB Biodisk, bioMérieux, Marcy l′Etoile, France), which yielded the exact MIC for every highly mupirocin-resistant isolate (MIC, ≥1,024 μg/ml).

Molecular biology analyses. The first step in the design of the mPCR was the selection of a variety of specific genes to identify at the species level clinical isolates from the six different staphylococcal species mentioned above and to detect high-level resistance to methicillin and/or mupirocin. The partially amplified loci are shown in Table 1. The primers selected for these amplifications had been previously described and were obtained from a commercial source (Integrated DNA Technologies, CA). For development of the mPCR, a DNA suspension from each isolate was rapidly prepared as previously described (19). Each primer pair was individually tested in a single PCR to ensure that the expected band was amplified (Fig. 1). Each of these single reactions was performed twice using DNA suspensions from two different isolates for each species. Moreover, some of the single PCRs have been used by us with collections of more than 200 *S. aureus* isolates, more than 100 *S. lugdunensis* isolates, and more than 50 *S. saprophyticus* isolates (unpublished data). The reproducible success we achieved using these primers made us choose them for developing the mPCR described herein. Furthermore, we expected that different pairs would yield fragments with different sizes (Fig. 1 and Table 1), which would facilitate their identification after the mPCR. Thus, each band was purified (Qiagen purification kit; Qiagen, CA) and the sequence determined in order to confirm the identities by comparison to NCBI data bank sequences. Sequencing of the amplicons was performed on an ABI-PRISM 310 genetic analyzer (Applied Biosystems Japan Co. Ltd., Tokyo, Japan) with BigDye terminator fluorescence chemistry (Applied Biosystems, Warrington, United Kingdom). In the case of the *S. hominis* isolate, the low prevalence of *mecA*-positive *S. hominis* clinical infections in our hospital suggested the convenience of molecular identification by sequencing its 16S rRNA genes. The *S. hominis* isolate 16S rRNA gene sequence had 99.9% identity with the *S. hominis* ATCC 27844 16S rRNA gene sequence (GenBank accession no. L37601.1). After band identity confirmation, the mPCR assay was optimized (Fig. 2), and the working protocol we used is described as follows. In a 25-μl reaction volume, 2.5 μl of a DNA suspension was used as the DNA template, and it was added to a 22.5-μl PCR mixture consisting of 1× reaction buffer, 0.2 mM each of the four deoxynucleoside triphosphates (dNTPs), 2.4 mM $MgCl_2$, 1 μM *nucA* primer pair, 0.5 μM *mvaA* primer pair, 0.5 μM *sep* primer pair, 0.5 μM *fbl* primer pair, 0.5 μM *sap* primer pair, 0.5 μM *mecA* primer pair, 0.5 μM *ileS2* primer pair, 0.5 μM *hom* primer pair, and 0.1 U/μl of *Taq* DNA polymerase (Biotherm DNA polymerase; Gene Craft, Germany). All mPCR assays were carried out with a negative control containing all reagents except the DNA template. DNA amplification was carried out in a GeneAmp PCR system 9700 thermocycler (PE Applied Biosystems, CA) with thermal cycling conditions consisting of an initial denaturization step at 94°C for 5 min, followed by 45 amplification cycles of (i) 10 cycles of denaturization at 94°C for 30 s, annealing at 64°C for 45 s, and extension at 72°C for 45 s; (ii) 10 cycles of denaturization at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min; and (iii) 25 cycles of denaturization at 94°C for 45 s, annealing at 52°C for 45 s, and extension at 72°C for 60 s, ending with a final extension step at 72°C for 10 min. After the mPCR, 4 μl from the reaction tube was subjected to agarose gel electrophoresis (2% agarose, 1× Tris-borate-EDTA, 8.5 V/cm, 75 min), using a 100-bp molecular size standard ladder (Roche, Basel, Switzerland) to estimate the sizes of the amplification products. The gel

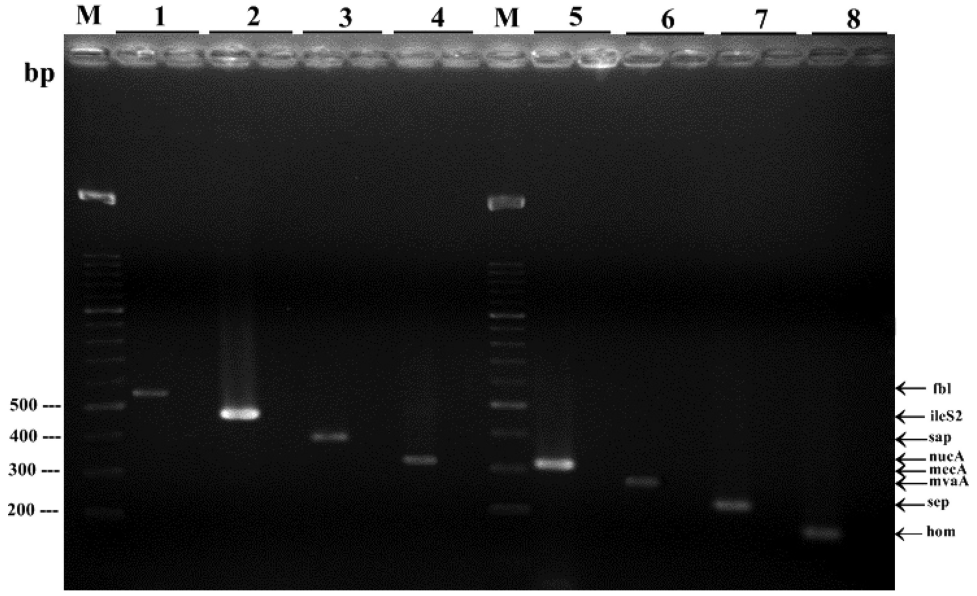

FIG 1 Agarose gel electrophoresis patterns showing single-PCR amplification products for *S. lugdunensis* gene *fbl* (lane 1), mupirocin resistance gene *ileS2* (lane 2), *S. saprophyticus* gene *sap* (lane 3), *S. aureus* gene *nucA* (lane 4), methicillin resistance gene *mecA* (lane 5), *S. haemolyticus* gene *mvaA* (lane 6), *S. epidermidis* gene *sep* (lane 7), and *S. hominis* gene *hom* (lane 8). Each pair of primers was amplified together with a negative control without DNA.

was stained with ethidium bromide, and the amplicons were visualized using a UV light in a GelDoc System (Bio-Rad, CA).

Method comparison studies. The concordance, efficiency, reproducibility, sensitivity, typeability, and discrimination power of the mPCR were estimated by use of bivariate ratios and the Simpson diversity index. Moreover, combined comparative analyses of gel images and phenotypic data were performed by using the Infoquest fingerprinting system, version 4.5 (Bio-Rad, CA).

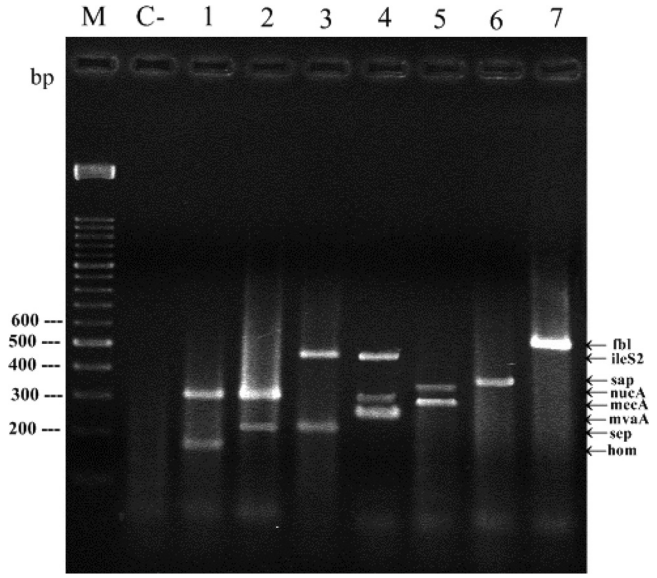

FIG 2 Agarose gel electrophoresis patterns showing mPCR amplification products from different staphyloccocal isolates. Lanes: C-, negative control without DNA; 1, methicillin-resistant *S. hominis* isolate (*hom* and *mecA* bands); 2, methicillin-resistant *S. epidermidis* isolate (*sep* and *mecA* bands); 3, mupirocin-resistant *S. epidermidis* isolate (*sep* and *ileS2* bands); 4: methicillin- and mupirocin-resistant *S. haemolyticus* isolate (*mvaA*, *mecA*, and *ileS2* bands); 5: methicillin-resistant *S. aureus* isolate (*nucA* and *mecA* bands); 6: methicillin- and mupirocin-susceptible *S. saprophyticus* isolate (*sap* band); 7: methicillin- and mupirocin-susceptible *S. lugdunensis* (*fbl* band).

Results. After the mPCR was performed for all 67 isolates, the *nucA* fragment amplified only in *S. aureus* strains and never in other staphylococcal isolates. Similarly, *fbl*, *mvaA*, *sap*, *sep*, and *hom* fragments yielded fragments only in *S. lugdunensis*, *S. haemolyticus*, *S. saprophyticus*, *S. epidermidis*, and *S. hominis* strains, respectively. As for the *mecA* fragment, it was detected in all strains that exhibited high methicillin resistance but not in the methicillin-sensitive ones. Similarly, amplification of the *ileS2* target always occurred for highly mupirocin-resistant strains, never for isolates with low or intermediate resistance, and never for the susceptible ones. The mPCR results for the isolates tested in this study are shown in Table 2.

After the 67 *Staphylococcus* isolates were analyzed with phenotypic, biochemical, and microbiological tools, single PCRs, and the newly described mPCR, the concordance of identification by classical methods with mPCR identification had a value of 1

TABLE 2 Staphylococcal clinical isolates, identification at the species level, susceptibilities to methicillin and mupirocin, and mPCR results

Species	No. of isolates[a]				
	Total	Mup^r	Met^r	Mup^r Met^r	Mup^s Met^s
S. aureus	31	2	19	5	5
S. epidermidis	14	3	2	0	9
S. hominis	1	0	1	0	0
S. saprophyticus	12	1	1	0	10
S. haemolyticus	3	0	1	2	0
S. lugdunensis	6	0	0	0	6
Total	67	6	24	7	30

[a] Mup, mupirocin; Met, methicillin; r, resistant; s, susceptible.

(100%), a sensitivity value of 1 (100%), and a specificity value of 1 (100%), a typeability value of 1 (100%), a reproducibility value of ≥0.95, and a discriminatory power of 0.9225 (http://insilico.ehu.es/mini_tools/discriminatory_power/index.php).

Concluding remarks. The clinical infections described here constitute health concerns, and therefore prompt identification of the staphylococcal infectious agents and the precise detection of their antibiotic resistances are crucial for successful management (7–9, 18–20). With these aims, we have developed a new single-tube multiple PCR assay that is very fast, extremely efficient, and sensitive. A limitation of this multiplex assay is that it can identify only *S. aureus* and the five above-mentioned coagulase-negative staphylococcal species. However, according to the literature (23), we have included the most clinically significant *Staphylococcus* spp., which are also the most frequently isolated in our hospital. Other species, such as *Staphylococcus schleiferi* and *Staphylococcus capitis*, have been rarely associated with endophthalmitis after surgery and neonatal sepsis, respectively, in some studies (17), but they have not caused complications in our hospital. Another possible limitation of the present study is the small number of methicillin- and/or mupirocin-resistant isolates we tested. But, as we have mentioned above, the larger number of isolates analyzed by single PCR reinforces the dependability of this multiplex PCR. In comparison with good previously described methods, such as the quadriplex PCR described by Zhang et al. (13), this new protocol has the advantage of obtaining species-specific amplicons, which permits species identification without the need for sequencing PCR fragments after the PCR. This protocol, from the preparation of cellular suspension to electrophoresis analysis of the PCR products on agarose gel, was performed in 5 to 6 h. The knowledge provided by the results obtained should dictate the appropriate antibiotic therapy in concert with preemptive measurements.

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